
Description

Single-stranded oligodeoxynucleotides containing CpG motifs
against influenza virus

FIELD

The present invention relates generally to a series of synthetic single-stranded oligodeoxynucleotides containing CpG motifs (CpG ODN) and especially to that having prophylactic and therapeutic effects on respiratory tract infections caused by influenza virus, such as human influenza. The present invention also relates to techniques and assumptions of utilizing synthetic single-stranded oligodeoxynucleotides containing CpG motifs to prevent and treat respiratory tract infections caused by influenza virus, such as human influenza.

BACKGROUND

Influenza virus is the pathogen of human influenza. 20,000,000 people worldwide died in the 1918-1919 influenza pandemic (Patterson, K. D. & Pyle, G. F. (1991) Bull. Hist. Med. 65, 4-213). The influenza virus being so fierce and unconquered closely relates to its own characteristics as follows:

- (1) Its easy spread by aerosol.
- (2) Its ability to escape from the forming protective immunity by

frequently changing its viral antigens (antigenic drift) (Parvin, J. D. , Moscona, A. , (1986) *J. Virol.* 59, 377-383 ; Webster, R. G., (1982) *Nature* 296, 115-121).

(3) The periodic emergence of new virulent strains by reassortment or mixing of RNA segments in the cells that simultaneously infected by different subtypes (Webster, R. G. , (1992) *Microbiol. Rev.* 56, 152-179).

Vaccines and antiviral drugs are two main methods to prevent and treat influenza (Bridges, C. B. , Fukuda, K. , Cox, N. J. & Singleton, J. A. (2001) *Morbid. Mortal. Wkly. Rep.* 50, 1-44). But in fact, these two methods could not control the epidemic of influenza effectively (Webby, R. J. & Webster, R. G. (2001) *Philos. Trans. R. Soc. London* 356, 1817-1828). In susceptible people, the protective efficacy of vaccination (6 months) is merely 39% (Fukuda, K. , N. J. (1999) *Morbid. Mortal. Wkly. Rep.* 48, 1-28, Castle, S. C. (2000) *Clin. Infect. Dis.* 31, 578-585). In addition, the existing vaccines have to be reformulated almost every year because the influenza viral antigens [hemagglutinin (HA) and neuraminidase (NA)] undergo frequent changes. Similarly, although antiviral drugs have been approved in the United States , their application is limited due to severe side effects and the possible emergence of resistant strains (Luscher-Mattli, M. (2000) *Arch. Virol.* 145, 2233-2248).

Recently, the studies have indicated that many bacterial or viral

DNAs containing CpG motifs act as “danger signals”, inducing activation of many kinds of immune cells to prime antiviral mechanisms of the immune system. “CpG” refers to the dinucleotide, where C is cytosine, G is guanine, and p represents the phosphodiester bond between the two. Further studies have indicated that synthetic oligodeoxynucleotides containing CpG motifs could also activate many kinds of immune cells to prime the antiviral mechanism of the organisms.

Vaginal epithelial application of CpG ODN in mouse and guinea pigs increased their resistance against lethal dose of HSV-2 virus challenge (Richard B. Pyles et al. *Journal of Virology*, November 2002, Vol.76, No. 22 p. 11387-11396). Administration of CpG ODNs also could reduce respiratory syncytial viral (RSV) load in a mouse model (Cho JY, et al. *J Allergy Clin Immunol* 2001 Nov;108 (5):697-702). And treating Friend virus(one kind of murine leukaemia virus)-infected mice with CpG ODN significantly reduced the viral loads in the blood (Anke R. M. Olbrich,et al. *Journal of Virology*, November 2002, Vol. 76, No. 22 p. 11397-11404). Furthermore, after treating C57BL/6 mice intravaginally with CpG ODN 2 days before an intravaginal challenge with normally lethal dose of herpes simplex virus type 2 (HSV-2), the results showed that CpG ODN treatment could significantly decrease the titers of HSV-2 in the vaginal fluids of the mice and stimulate the genital tract mucosa to secrete protective cytokines, such as IFN- γ , IL - 12, IL - 8 (Harandi AM, et al. *J*

Virol 2003;77(2):953).

Contents of the invention

Overview

The first objective of this invention provides a series of synthetic oligodeoxynucleotides including cytidylyl -3'-5'-guanosine(CpG ODN), especially those that can stimulate peripheral blood cells to resist influenza virus. They are composed of oligodeoxynucleotides including one or more CpG sequences. The oligodeoxynucleotides may be phosphorothioate-modified partly , completely or unmodified. The optimizing CpG ODN sequences are showed in SEQ ID NO : 1-5.

The second objective of this invention provides the effect of the synthetic CpG ODN on resisting influenza virus by.

The third objective of this invention provides the prophylactic and therapeutic effects of the synthetic CpG ODN on resisting respiratory tract infections, such as the epidemic of influenza caused by influenza virus

The forth objective of this invention provides the prophylactic and therapeutic techniques and assumptions of synthetic CpG ODN resisting infectious diseases of respiratory tract, such as infectious disease of respiratory caused by influenza virus.

In addition, it should be known that the substantial features and

creative effects besides the public context of this application can be deduced directly by common technologist of this field.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

In the context of this invention, the terms are well known to those skilled in the art, except for additional explanations. Specifically, the following terms have the meanings below:

The optimized single-stranded oligodeoxynucleotides containing CpG motifs according to the present invention have the sequences listed below:

DVAX-1: 5'-TCgTCgggTgCgACgTCgCAggggggbelow

DVAX-3: 5'-TCgTCgTTTCgTCgTTgggg-3'

DVAX-4: 5'-TCgACgTTCgTCgTTCgTCgTTC-3'

DVAX-5: 5'-TCggggACgATCgTCgggggg-3'

DVAX-6: 5'-ggATCgATCgATCgATgggggg-3'

The CpG ODNs may be phosphorothioate-modified partly, completely or unmodified.

The CpG ODN in this invention can be produced by techniques well known in the art, for example, solid phase phosphoramidite trimer method. The following embodiments illustrate an exemplary method of

producing such compositions according to the invention in detail.

The dose of these synthetic CpG ODNs is 1-5000 µg at one time for preventing and treating respiratory tract infections caused by SARS virus.

These synthetic CpG ODNs in this invention can be applied alone, by two or more kinds of them combination, by mingling with drugs or vaccines that can treat or prevent infectious diseases caused by influenza virus, by covalent coupling with drugs or vaccines mentioned above through cross linking agents.

These synthetic CpG ODNs in this invention can be administered through mucosa (including respiratory tract, digestive tract, genitourinary tract), by ophthalmic preparation, by subcutaneous and intramuscular injection, by stomach intestine, by intraperitoneal injection, by intravenous injection.

This invention is described in detail through the examples of the preparation and biological effects and accompanying figures. It should be understood that these instances are for purposes of illustration only and are not intended to limit the invention by any manner of means.

Example

Some procedures in embodiments below, which are not given a detailed description, are routine methods known in this field, such as solid phase phosphoramidite trimer method. The sources, trade names

and/or some necessary ingredients of the agents are labeled only one time. Subsequently, the identical agents will not be labeled unless special explanations are given.

Example 1

Preparation of the Synthetic Single-Stranded Oligodeoxynucleotides Containing CpG motifs

Solid phase phosphoramidite trimer method was used for synthesis of the single-stranded oligodeoxynucleotides containing CpG motifs

1. Reagents and Materials

Trichloroacetic Acid(TCA), Controlled Pore Glass (CPG) , DMT, Tetrazole activator, Acetic anhydride, N-methylimidazole, Deoxynucleoside triphosphate (dNTPs including A, T, C and G), ABI DNA synthesizer, Sensitive Liquid Chromatograph, etc.

2. Methods

Deblocking

Deblocked off the DTM (a nucleotide blocking group) from controlled pore glass (CPG) by trichloroacetic acid (TCA) to obtain a free 5'-OH group for condensation reaction.

Activation

Put the phosphoramidite protected nucleotide monomers and tetrazole activator mixture into composite column together to get a phosphoramidite tetrazolium reactive intermediate. This intermediate (activation in the 3' terminal, and DMT protection in the 5' terminal) would be condensed with deblocked nucleotides on controlled pore glass (CPG).

Coupling

When encountered with the deblocked nucleotides on controlled pore glass (CPG), phosphoramidite tetrazolium reactive intermediate would bind with their 5'-OH and condensate to deblock off tetrazolium, then the synthetic oligonucleotides extended forward for one basic radical.

Capping

To prevent the extension of nonreacted 5'-OH on controlled pore glass in the sequential circulations after condensation reaction, we often capped this 5'-OH by acetylation. Generally, the acetylation reagent was generated by mixing acetic anhydride and N-methylimidazole.

Oxidation

Nucleotide monomers were connected with oligomers on the controlled pore glass by phosphite bonds in condensation reaction. But phosphite bonds were instable and prone to be hydrolyticed by acid and base, therefore we often transformed phosphoryl into phosphotriester with iodine tetrahydrofuran solution to obtain stable oligomers.

After the five procedures above, a dextyornucleotide was linked to the nucleotides on controlled pore glass. Samely, deblocked off the DTM (a nucleotide blocking group) from the latest dextyoneclotides 5'-OH by trichloroacetic acid and repeated the activation, coupling, capping and oxidation, we obtained a coarse DNA fragment. Finally, the coarse DNA fragment would be cutted, deblocked (benzoyl protection in A and C, isobutyryl protection in G, no protection in T, and phosphorous acid is protected by nitrile ethyl), purified (such as HAP, PAGE, HPLC, C18, OPC, etc.) and quantified etc, then oligonucleotide fragments according to experimental requirments would be obtained.

Nonphosphorothioate-modified single-stranded oligodeoxynucleotides containing CpG motifs were synthesized on ABI 3900 DNA synthesizer; completely or partly phosphorothioate-modified single-stranded oligodeoxynucleotides containing CpG motifs were synthesized on ABI 394 DNA synthesizer by displacement method.

Example 2

**Anti Influenza Virus Activity of Synthetic Single-Stranded
Oligodeoxynucleotides Containing CpG motifs**

1. Isolation of Human PBMC (peripheral blood mononuclear cell)

Instruments and Equipments

Cryogenic refrigerator, CO₂ Incubator, Clean bench, Inverted microscope, Liquid nitrogen biological container, Water distiller, Vacuum pump, Culture vessels, Filter, Filter flask, Straws (different standards), Pipette, Dropper, Blood count chamber, Horizontal centrifuge, etc.

Reagents and materials

Heparin anticoagulated human whole blood from two healthy volunteers
Changchun Blood Center

Ficoll-Urografin

Specific gravity: 1.077 ± 0.001 , Beijing DingGuo Biotechnology Company
Limited

IMDM: 1000 mL IMDM contain 100000 U gentamycin were filtered by 0.22 μ m diameter membrane to sterilize and then divided equally to small volume.

Methods

Human PBMCs (peripheral blood mononuclear cells) were isolated by Ficoll-Hypaque. The volume of Ficoll-Urografin was twice of the Heparin anticoagulated human whole blood. After horizontally centrifuging (1000 g) for 20 min, the layer of mononuclear cells was isolated and transferred to another centrifuge tube. The same volume IMDM without serum were added and then centrifuged (1000 g) for 15 min. The supernatants in centrifuge tube were discarded. The cells were washed twice and the supernatants were discarded again. Finally resuspended the cells with 2 mL IMDM and counted the cell number.

2. Procedure for Testing the Effect of Synthetic Oligodeoxynucleotides Containing CpG Motifs on Anti-influenza Virus

Human PBMCs were suspended in IMDM containing 10% fetal bovine serum (FBS) at a concentration of 3×10^6 cells/ml. The cells were then seeded in 12-well plates (2 ml/well). The synthetic oligodeoxynucleotides containing CpG motifs (CpG ODNs) were added at a concentration of 6.25 μ g/ml. The medium group without CpG ODNs and GpC ODNs (DVAX-1) were as control groups, respectively. The sequence of DVAX-1 was 5'-TgCTTgggTggCAgCTgCCAgggggg-3'. After 48 h culture in a 5% CO₂ humidified incubator at 37 °C, the supernatants were collected for evaluating their abilities of anti-influenza

viruses.

Viable Vero E6 cells were suspended in IMDM medium containing 5% FBS at a concentration of 3×10^5 cells/ml. The cells were then seeded in 96-well plates (100 μ l/well). CpG ODNs stimulating supernatants and control supernatants were added (100 μ l/well) in a dilution of 1:10 by 5% FBS IMDM (The final volume became 200 μ l/well, i.e. suspended cells 100 μ l plus diluted CpG ODN stimulating supernatants or control supernatants 100 μ l). The cells were incubated in a 5% CO₂ humidified incubator at 37°C for 24 hours. Then the medium was discarded and the cells were incubated continuously with 200 μ l/well of the diluted influenza viruses (Institute of Biological Products, ChangChun). The interferon- α , influenza viruses and Vero E6 cells alone were as control groups, respectively. After 48 or 72 hours culture, when 80%-100% Vero E6 cells of influenza virus control group were fully cytopathic, while cells of Vero E6 cells control group were still viable, the culture was terminated. The cytopathic effects were evaluated by Methylrosaniline Chloride Staining Assay. Briefly, the medium was discarded and 0.5% methylrosaniline chloride staining solution was added (200 μ l/well). After incubation for 15 minutes in 37 °C, the methylrosaniline chloride staining solution was washed by flowing water.

0.5% Methylrosaniline Chloride Staining Solution: Methylrosaniline Chloride 0.5g and Sodium Chloride 0.85g were dissolved by 50ml

anhydrous ethanol and then 3ml methanol and 47ml distilled water were added.

The results were recorded by photographs. Vero E6 cells were seeded in 96-well plate as shown in figure 1. The results of methylrosaniline chloride staining assay were indicated in figure2.

Figure-1: Table for addition of the samples in 96-well plates

DVAX-1 control: synthetic oligodeoxynucleotides containing GpC motifs, the sequence was 5'-TgCTTgggCAgCTgCCAgggggg-3';

Medium control: supernatants of human PBMCs without CpG ODNs stimulation;

Vero E6 cells control: influenza viruses were not added;

Influenza virus control: influenza viruses were added without stimulating supernatants.

DVAX-1, 3, 4, 5, 6 used herein represent synthetic single-stranded oligodeoxynucleotides containing CpG motifs whose codes are DVAX-1, 3, 4, 5, 6 respectively.

IFN- α control: interferon- α (Institute of Biological Products, ChangChun) was added with influenza viruses;

C, D, E1-10; F, G, H3-4: stimulated supernatants from one donor's PBMCs;

C, D, E11-12; F, G, H5-12: stimulated supernatants from another donor's PBMCs;

Figure-2: Anti-influenza virus effects of the supernatant of CpG ODN-stimulated human PBMCs.

The results indicated that the synthetic oligodeoxynucleotides containing CpG motifs DVAX-1, DVAX-3, DVAX-4, DVAX-5 and DVAX-6 (SEQ ID NO: 1-5) could stimulate human PBMCs to secrete some antiviral substances, which could significantly protect Vero E6 cells from influenza viruses. That predicted the synthetic oligodeoxynucleotides containing CpG motifs DVAX-1, DVAX-3, DVAX-4, DVAX-5 and DVAX-6 (SEQ ID NO: 1-5) have therapeutic and prophylactic effects on infectious diseases of respiratory tract, such as flu caused by influenza viruses.

SEQUENCE LISTING

<110> Changchun Huapu biotech Co.,Ltd.

<120> Single-stranded oligonucleotides containing CpG motifs against influenza virus

<160> 6

<210> 1

<211> 26

<212> DNA

<213> artificial

<220>

<221>

<222>

<223>

<400> 1

tcgtcgggtg cgacgtcgca gggggg

26

<210> 2

<211> 20

<212> DNA

<213> artigicial

<220>

<221>

<222>

<223>

<400> 2

tcgtcgtttcgtcgttgggggg

20

<210> 3

<211> 20

<212> DNA

<213> artigicial

<220>

<221>

<222>

<223>

<400> 3

tcgtcgtttcgtcgttgggg

20

<210> 4

<211> 23

<212> DNA

<213> artigicial

<220>

<221>

<222>

<223>

<400> 4

tcgacgttcgtcgttcgtcgttc

23

<210> 5

<211> 21

<212> DNA

<213> artigicial

<220>

<221>

<222>

<223>

<400> 5

tcggggacgatcgtcggggggg

21

<210> 6

<211> 22

<212> DNA

<213> artigicial

<220>

<221>

<222>

<223>

<400> 6

ggatcgatcgatcgatgggggg

22

Claims

1. Synthetic single-stranded oligodeoxynucleotides containing one or more CpG motifs.
2. The oligodeoxynucleotides of claim 1, wherein the phosphodiester bonds can be partly or completely phosphorothioate-modified, or unmodified.
3. The oligodeoxynucleotides of claim 1 comprising a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5.
4. The oligodeoxynucleotides of claim 1,2,3 induce cell to secrete materials against infection of influenza virus.
5. The oligodeoxynucleotides of claim 1,2,3 treat respiratory tract infections caused by influenza virus, such as human influenza.
6. The techniques and assumptions of using the oligodeoxynucleotide of claim 1,2,3 to treat respiratory tract infections caused by influenza virus, such as human influenza.

Abstract

CpG-containing single-stranded oligodeoxynucleotides against influenza virus

The invention provides a series of artificial CpG-containing single-stranded oligodeoxynucleotides (ODNs), each of which is consisted of single-stranded oligodeoxynucleotide DNA molecule containing one or more CpG(s), wherein the ODNs can stimulate human peripheral blood mononuclear cell (PBMC) to produce the substances against influenza viruses. The substances can protect cell from damaging of influenza virus infection. Therefor, those CpG containing single-stranded oligodeoxynucleotides show a function of resisting influenza viruses and can be use for preventing or treating human respiratory tract infections caused by influenza viruses.

Fig 1:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Culture medium control		IFN(10IU/ml)		IFN(100IU/ml)		IFN(1000IU/ml)		Inflenza virus		Normal Vero cell	
B	Normal Vero cell		IFN(10IU/ml)		IFN(100IU/ml)		IFN(1000IU/ml)		Normal Vero cell		Normal Vero cell	
C	0.125ug/ml	DVAX-3	0.125ug/ml	DVAX-1	0.125ug/ml	DVAX-1	DVAX-5	DVAX-5	DVAX-6	DVAX-6	DVAX-1	DVAX-1
	1:20 dilute		1:20 dilute		1:20 dilute		1:20 dilute		1:20 dilute		1:20 dilute	
	6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml	
D	0.125ug/ml	DVAX-3	0.125ug/ml	DVAX-1	0.125ug/ml	DVAX-1	DVAX-5	DVAX-5	DVAX-6	DVAX-6	DVAX-1	DVAX-1
	1:100 dilute		1:100 dilute		1:100 dilute		1:100 dilute		1:100 dilute		1:100 dilute	
	6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml	
E	0.125ug/ml	DVAX-3	0.125ug/ml	DVAX-1	0.125ug/ml	DVAX-1	DVAX-5	DVAX-5	DVAX-6	DVAX-6	DVAX-1	DVAX-1
	1:500 dilute		1:500 dilute		1:500 dilute		1:500 dilute		1:500 dilute		1:500 dilute	
	6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml	
F	DVAX-3	DVAX-3	DVAX-1	DVAX-1	DVAX-1	DVAX-1	DVAX-4	DVAX-4	DVAX-5	DVAX-5	DVAX-6	DVAX-6
	1:20 dilute		1:20 dilute		1:20 dilute		1:20 dilute		1:20 dilute		1:20 dilute	
	6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml	
G	DVAX-3	DVAX-3	DVAX-1 control	DVAX-1 control	DVAX-1 control	DVAX-1 control	DVAX-4	DVAX-4	DVAX-5	DVAX-5	DVAX-6	DVAX-6
	1:100 dilute		1:100 dilute		1:100 dilute		1:100 dilute		1:100 dilute		1:100 dilute	
	6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml	
H	DVAX-3	DVAX-3	DVAX-1 control	DVAX-1 control	DVAX-1 control	DVAX-1 control	DVAX-4	DVAX-4	DVAX-5	DVAX-5	DVAX-6	DVAX-6
	1:500 dilute		1:500 dilute		1:500 dilute		1:500 dilute		1:500 dilute		1:500 dilute	
	6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml		6.25ug/ml	

Fig 2:

